Immunogenomic Approaches to Study Host Immunity to Enteric Pathogens[1]

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ABSTRACT With increasing consumer demands for safe poultry products, effective control of disease-causing pathogens is becoming a major challenge to the poultry industry. Many chicken pathogens enter the host through the gastrointestinal tract, and over the past few decades, in-feed antibiotics and active vaccination have been the 2 main mechanisms of disease control. However, increasing public concerns are prompting government regulations on the use of growth-promoting drugs in animal production, and the ability of current vaccines to protect against emerging hypervirulent strains of pathogens is becoming an issue. Therefore, there is a need to develop alternative control strategies against poultry pathogens of economic importance as well as to carry out basic research to enhance understanding of host-pathogen interactions at local sites of infection. Effective control strategies against pathogens can only be accomplished by comprehensive analysis of the basic immunobiology of host-pathogen interactions. Recent sequencing of the poultry genome and the availability of several tissue-specific cDNA microarrays are facilitating the rapid application of functional immunogenomic technologies to poultry disease research. Studies using functional genomic, immunology, and bioinformatic approaches have provided novel insights into disease processes and protective immunity to chicken pathogens. In this review, we summarize recent published literature concerning the host response to *Eimeria* and *Salmonella* infections with emphasis on our studies using immunogenomic tools to investigate and characterize the mechanisms of avian immunity to these mucosal pathogens. The results clearly indicate that this immunogenomic approach will lead to increased understanding of immune responses to infectious agents that will enable the development of effective prevention strategies against mucosal pathogens.

Key words: pathogen, immunogenomic tool, *Eimeria*, *Salmonella*

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CHICKEN IMMUNE RESPONSES TO *EIMERIA*

Coccidiosis is a poultry disease of substantial economic importance, estimated to cost the US industry greater than $700 million annually. In the absence of efficient vaccines to control coccidiosis and the emergence of new antigenic variants of field stains of *Eimeria*, the broiler industry has relied upon prophylactic medication. However, anticoccidial drugs are expensive, and their effectiveness is hindered by widespread incidence of drug resistance and the high cost of new drug development (Chapman, 1998). Moreover, there are increasing government regulations on the use of in-feed antibiotic growth promoters, and most nontherapeutic antibacterial feed additives have been banned in the European Union since 1999 (European Union legislation, Council Regulation 2821/98). Therefore, it is of interest to develop alternative control strategies for coccidiosis (Dalloul and Lillehoj, 2005). In this regard, understanding the complex nature of the interaction among various factors controlling protective immunity to *Eimeria* is critical (Lillehoj et al., 2004; Dalloul and Lillehoj, 2005).

Examination of the patterns of disease resistance following experimental coccidiosis has suggested 2 separate mechanisms of protective immunity: innate immunity during initial pathogen exposure (primary infection) and acquired immunity following secondary infection (Lillehoj et al., 1999). In immune chickens, parasites enter the gut early after infection but are prevented from further development, indicating that acquired immunity to coccidiosis may involve mechanisms that inhibit the natural progression of parasite development (Trout and Lillehoj, 1996). Although a direct role of immune effector lymphocytes in inhibiting parasite development has not been demonstrated, $CD8^+$ cytotoxic T cells and interferon-γ

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(**IFN-γ**) have been identified as key components of the host protective immune response (Lillehoj and Choi, 1998; Lillehoj et al., 2004). At the genetic level, both MHC-linked genes and non-MHC genes have been implicated in controlling host immunity to coccidiosis (Lillehoj et al., 1989), and our recent study demonstrated the existence of a quantitative trait locus controlling disease resistance located on chicken chromosome 1 (Zhu et al., 2003; Kim et al., 2006).

The development of novel strategies to control coccidiosis will only be realized after completion of a systematic and detailed analysis of local host-parasite interactions at the molecular and cellular levels. In particular, there is a need to advance fundamental knowledge of the basic immunobiology of the events associated with parasite invasion and intracellular development, as well as parasite biology and metabolism. Immune responses to coccidia are extremely complex, with different effector mechanisms playing roles at various stages of parasite development. The level of susceptibility to coccidiosis depends on a variety of factors including the particular *Eimeria* species, prior host exposure, the nutritional status of infected chickens, and the genetic makeup of the host (Lillehoj et al., 2004), and in many of these areas, we are only beginning to scratch the surface of elucidating their roles in *Eimeria* infection.

One area that has received considerable attention in recent years is cell-mediated immunity to coccidiosis, particularly with respect to the cloning and analysis of chicken cytokines and chemokines. Cytokines mediate intercellular signals during normal immune responses and have been investigated as potential vaccine immunopotentiators for avian coccidiosis (Lillehoj and Lillehoj, 2000; Lillehoj et al., 2004). Most chicken cytokines homologous to their mammalian counterparts have been described (Kaiser et al., 2000; Hong et al., 2006b), of which IFN-γ has shown promise as an immunomodulator (Yun et al., 2000). The chicken IFN-γ gene has been cloned (Song et al., 2000), transfected into chicken fibroblast cells, and shown to inhibit intracellular development of *Eimeria tenella* following in vitro infection (Lillehoj and Choi, 1998). An identical effect on *E. tenella* development in vivo has been observed after administration of recombinant IFN-γ protein to chickens before challenge with virulent parasites. Recently, a 19-kDa recombinant *Eimeria acervulina* protein (3-1E) stimulating IFN-γ production by chicken spleen cells was identified as profilin. Recombinant *Eimeria* profilin expressed in bacterial and eukaryotic vectors has been shown to induce protective immunity against live challenge infection when injected into young chickens (Lillehoj et al., 2000). Coadministration of recombinant profilin with cDNA encoding chicken IFN-γ or interleukin- (**IL**) 15 has been shown to lead to further enhancement of *Eimeria*-specific immunity.

At the level of the host response to coccidiosis, a novel peptide secreted by T and natural killer (**NK**) cells with antiparasitic activity has been identified as NK lysin (Hong et al., 2006a). Natural killer lysin is an antimicrobial and antitumor protein, and a full-length cDNA encoding chicken NK lysin has been isolated from a library prepared from chicken intestinal intraepithelial lymphocytes (**IEL**). Quantitative reverse transcription-PCR (**RT-PCR**) analysis of NK lysin gene expression has revealed high levels of transcripts in the intestinal IEL and spleen cells but low levels in thymic lymphocytes. Following infection with *Eimeria maxima*, NK lysin transcript levels have been shown to increase 3- to 4-fold in $CD4^+$ and $CD8^+$ intestinal IEL. Recombinant chicken NK lysin expressed in COS7 cells have been shown to exhibit antitumor cell activity against LSCC-RP9 tumor targets and have been directly cytotoxic for sporozoites of *E. acervulina*.

Recent development of functional genomic technologies and the availability of tissue-specific chicken cDNA microarrays have facilitated a more comprehensive investigation of host-pathogen interactions during avian coccidiosis (Min et al., 2003; Hong et al., 2006b,d). New candidate genes that influence host immune response to *Eimeria* have been identified using global gene expression analyses of chicken macrophage and intestinal IEL cDNA microarrays (Min et al., 2005; Hong et al., 2006a,c). These new developments in our understanding of host-pathogen immunobiology in avian coccidiosis raise the exciting possibility of developing novel control strategies against coccidiosis.

CHICKEN IMMUNE RESPONSES TO *SALMONELLA*

Salmonella is a gram-negative, aerobic, noncapsulated, nonsporulating, motile bacillus that infects amphibian, avian, and mammalian hosts. Salmonellosis is one of the most important foodborne zoonotic diseases throughout the world, and poultry represent an important source of infection in man. Among the 2,200 serovars of *Salmonella*, *Salmonella enterica* serovar Enteritidis (*Salmonella* Enteritidis) and Typhimurium (*Salmonella* Typhimurium) are responsible for the majority of foodborne enteritis in humans. Approximately 4% of the US population suffers from at least 1 case of food poisoning each year, with an estimated cost of almost $6 billion. During the last decade, many strategies have been applied in an effort to reduce *Salmonella* contamination on commercial poultry farms. These include use of antibiotics and other chemical feed supplements that inhibit *Salmonella* adhesion in the intestine, competitive exclusion by nonpathogenic bacteria, genetic selection of chicken strains for improved immune responses, and development of *Salmonella* vaccines (McHan et al., 1991; Bailey et al., 1998; Kaiser et al., 2000).

Salmonella enterica infection in chickens triggers both humoral and cell-mediated immune responses. Immunoglobulin G, IgA, and IgM produced in the intestinal mucosa and serum, $CD4^+$ T and IgG^+ B cells in cecal tonsils, and IgA^+ and IgM^+ B cells and $CD4^+$ T cells in spleen are all increased during experimental salmonellosis in chickens (Hassan et al., 1991; Brito et al., 1993; Sasai et al., 1997, 2000). Interestingly, $CD8^+$ T cells are decreased in the cecal tonsils and spleen after primary infection but not following secondary challenge infection with *Salmo-*

nella (Sasai et al., 1997; Babu et al., 2003). Additional changes in immune cells have been observed in the ovary and oviduct following primary and secondary infections with *Salmonella* Enteritidis. The number of macrophages and T cells in ovary and oviduct epithelium have been shown to increase significantly by d 7 postinoculation, peak at d 10, and return to preinoculation levels by d 21. The increases in macrophages and T-cell proliferation have been shown to immediately precede a decline in the number of *Salmonella* Enteritidis-positive tissues (Withanage et al., 1998, 2003). The correlation of elevated lymphocytes and macrophages with decreased *Salmonella* Enteritidis recovery suggests that local cell-mediated immunity is involved in controlling *Salmonella* Enteritidis colonization. Interleukin-1, IL-6, IL-8, and transforming growth factor-β4 are highly induced, whereas IL-18 and IFN-γ are downregulated in chicken heterophils or in immune organs with *Salmonella* Enteritidis infection (Beal et al., 2004). A CXC chemokine, K60, and macrophage inflammatory protein-1β (**MIP-1β**) have been upregulated in the intestine and liver (Withanage et al., 2004), and increased levels of other cytokines such as CCLi2, CXCLi2, IL-10, IL-12α, and IL-12β also have been demonstrated in *Salmonella* Enteritidis-infected chickens (Cheeseman et al., 2007).

In mammals, macrophage recognition of *Salmonella* is carried out through the cell surface pathogen-associated molecular patterns such as toll-like receptors (**TLR**) and CD14. Toll-like receptor 4 binds to the lipopolysaccharide (**LPS**) of gram-negative bacteria (Takeuchi et al., 1999) and activates macrophages through an intracellular signaling pathway that results in the induction of various proinflammatory cytokines (e.g., IL-6) and cytotoxic molecules (e.g., NO). In chickens, macrophages express TLR4 and CD14 (Dil and Qureshi, 2002), and TLR4 has been linked to a *Salmonella* Enteritidis-resistant phenotype in broilers (Leveque et al., 2003). Toll-like receptor 5, which recognizes bacterial flagella, has also been described in chickens (Hayashi et al., 2001), and its expression has been associated with IL-1β production (Iqbal et al., 2005). Chicken TLR15 is a newly identified pathogen-associated molecular patterns receptor, and its expression in the cecum of *Salmonella* Typhimurium-infected chickens suggests a role in host defense against bacteria (Higgs et al., 2006).

Different serovars of *Salmonella* use different survival strategies to circumvent host immunity, but the underlying mechanisms of immune evasion in avian salmonellosis are not well understood. For instance, *Salmonella* Typhimurium infection of chickens induces lymphocyte depletion and atrophy of lymphoid organs that may have a critical role in *Salmonella* pathogenesis (Hassan and Curtiss, 1994). Furthermore, *Salmonella* Enteritidis invasion downregulates IL-1 and IL-2 production, whereas *Salmonella* Gallinarum infection does not induce any inflammatory cytokine response including IL-1, IL-2, or IL-6 (Kaiser et al., 2000). Although the underlying mechanisms that *Salmonella* utilizes to evade protective host immune responses need to be better identified, *Salmonella* vaccines have shown promise in controlling the infection in poultry. For example, following vaccination of chickens with killed *Salmonella* Enteritidis, increased splenic lymphocyte proliferation and serum IL-2 and IL-6 levels were observed by Okamura et al. (2003, 2004). Protective immune response to vaccination depends on several variables, including age: Four-week-old chickens have shown higher lymphoproliferation response to *Salmonella* Enteritidis LPS and flagella compared with 8-mo-old birds. Younger chickens have also produced higher levels of IFN-γ and IL-2 by antigen-stimulated splenocytes after vaccination with an inactivated *Salmonella* vaccine compared with older animals, but the vaccination has been shown to induce higher cytokine production regardless of age. Furthermore, splenocytes from vaccinated chickens have shown significantly increased numbers of TCR$\gamma\delta^+$ cells at 7 d postvaccination compared with nonvaccinated controls, although no significant differences in the number of $CD4^+$, $CD8^+$, or TCR$\alpha\beta^+$ cells was observed. Higher levels of *Salmonella* Enteritidis flagella-induced NO production have been observed at 4, 7, 11, and 14 d postvaccination, and serum IFN-γ, IL-1, IL-6, and IL-8 have been shown to be elevated at 7 d following *Salmonella* vaccination. These results indicate that younger chickens demonstrate a more robust antigen-specific immune response to the *Salmonella* Enteritidis vaccine compared with older birds, and vaccination induces a T-cell–mediated proinflammatory response. However, further studies are necessary to obtain a more comprehensive understanding on host-bacteria interaction in avian salmonellosis.

APPLICATION OF FUNCTIONAL GENOMICS TO COCCIDIOSIS

Complete sequencing of its genome together with the availability of various tissue-specific microarrays has opened the field of large-scale functional genomic analyses of chickens. Among these approaches, DNA microarray technology, which allows high throughput measurement of global gene transcription on a whole-genome or tissue-specific basis, now enables the investigation of the transcription status of complex biological systems. Specifically, genomics technology combined with immunology (immunogenomics) allow in-depth analysis of complex immunological processes based on large-scale genomic approaches.

Deoxyribonucleic acid arrays have become popular, because they are generally considered easier to use compared with the other gene expression profiling methods, and they allow the parallel quantification of thousands of genes from multiple samples. Gene expression analysis using microarrays has become a powerful tool to evaluate the complexity of host-pathogen immunobiology. Conceptually, DNA array technologies rely on nucleic acid hybridization between labeled free targets derived from a biologic sample and an array of many DNA fragments (the probes, representing genes of interest), tethered to a solid surface. The targets, often produced by reverse transcription of mRNA and simultaneous labeling of the

corresponding cDNA, form a complex mixture of fragments that hybridize with their cognate probes during the assay. The signal generated on each probe reflects the mRNA expression level of the corresponding gene in the sample. After detection, quantification, and integration of signals with specialized software, intensities are normalized for technical deviations, providing a gene expression profile for each sample comparable to profiles from other samples.

Although a variety of large-scale commercial microarrays are available for human and other mammalian species, there are few such tools available for agricultural species. In chickens, a limited number of low- and high-density cDNA microarrays have been developed (Morgan et al., 2001; Min et al., 2003; Neiman et al., 2003; Cogburn et al., 2004; Bliss et al., 2005). More recently, a consortium of research groups developed a 13,000-element chicken cDNA microarray, and there is a commercially available whole-genome chicken oligonucleotide array (Affymetrix Corp., Sunnyvale, CA) for use by the avian research community. Our own studies have focused on the development of a chicken intestinal IEL array (**CIELA**) that contains >10,000 EST selected from a normalized chicken cDNA library. Included in this array are chemokine, cytokine, and lymphokine elements, TLR, IFN-antiviral response pathway genes, genes involved in the oxidative burst response, apoptosis-related genes (including perforin and granzyme B), and a variety of genes encoding surface markers for monocytes, macrophages, heterophils, dendritic cells, and T cells. In addition, 2 tissue-specific chicken cDNA microarrays with about 5,000 and 10,000 preselected EST from macrophages (Bliss et al., 2005) and intestine IEL (Min et al., 2005), respectively, have been used to investigate local gene expression profiles and host innate and adaptive immune responses of broiler chickens infected with *Eimeria*.

The avian macrophage microarray (**AMM**) and CIELA have been successfully applied to different avian cell or tissue types as well as a variety of pathogens, and both have proven to be useful tools to identify global gene expression changes. For example, changes in expression of genes involved in innate immunity have been observed in vivo from experimentally infected chickens and in vitro using peripheral blood monocytes, heterophils, nonadherent blood lymphocytes, and multiple avian macrophage cell lines. In addition, innate immune responses have been elucidated following stimulation with *Salmonella*, *Escherichia coli*, *Mycoplasma*, influenza viruses, *Eimeria*, cellular components (LPS), and immune modulators (IFN-γ) using these arrays. Use of the AMM has led to better understanding of the innate immune response mediated by avian macrophages in response to 3 antigenically distinct species of *Eimeria*, *E. acervulina*, *E. maxima*, and *E. tenella* (Dalloul et al., 2007). In a study by Dalloul et al. (2007), a set of core response elements was identified comprising 25 genes, including many immune-related genes, whereas 60 to 67 elements were uniquely induced or repressed by the individual species. Such differential responses may be attributed to the species-specific immunity induced by different *Eimeria* species, and a deeper look into the functional aspects of these elements may lead to the elucidation of the pathogenicity, immunogenicity, or both, of each species and better design of a coccidiosis vaccine.

One may ask, "Why is there a need to determine the similarities and differences in host immune responses induced by *Eimeria* species?" *Eimeria acervulina*, *E. maxima*, and *E. tenella* are the most common coccidia encountered in the field, and each infects a unique site in the chicken intestine. Infections, when not deadly, induce protective immunity against subsequent challenges; however, such immunity remains confined to homologous species with no cross-species protection (Lillehoj et al., 2004). Among the 3, *E. maxima* infection is characterized by high immunogenicity, in which priming infection with few oocysts induces full protective immunity to subsequent homologous challenge. Conversely, far more *E. acervulina* and *E. tenella* oocysts are required to induce comparable protective immunity. For these reasons, identification of the early host responses at the gene transcription level provides a molecular immune profile of the events that occur during and immediately following infection with *Eimeria*.

The total number of unique elements exhibiting significant expression changes using the AMM is shown in Table 1. Table 2 summarizes the response of several immune-related genes following infection of chickens with *E. acervulina*, *E. maxima*, and *E. tenella*. Tables 3 and 4 list the 20 genes demonstrating the greatest increases or decreases in expression following infection with *E. maxima*. Of the 25 core response elements that have been induced by all 3 *Eimeria* are several important immune effector genes, such as the proinflammatory cytokine IL-1β, the chemokines ah221 and MIP-1β, and osteopontin (Table 2). By quantitative RT-PCR, IL-1β has been shown to be highly induced (>5-fold) by the 3 *Eimeria* parasites. Rodenburg et al. (1998) showed that IL-1β is secreted by macrophages and other cells upon activation by a variety of different stimuli. In turn, IL-1β upregulates the production of other chemokines including MIP-1β, K203, and ah221 and cytokines such as osteopontin, thereby amplifying the immune response. Furthermore, MIP-1β and K203 belong to the CC chemokine family, normally involved in the recruitment of macrophages. Using IFN-γ-stimulated HD11 chicken macrophages, Laurent et al. (2001) observed similar results, suggesting that macrophages are the main effector inflammatory cells at *Eimeria* infection sites. Osteopontin has been described as an important component of early cellular immune responses (Patarca et al., 1993). It is known to directly induce chemotaxis and indirectly facilitate macrophage migration to other chemoattractants and has been characterized as an early protein expressed by activated macrophages and NK cells (O'Regan et al., 2000). Osteopontin enhances T helper (**Th**) 1 and inhibits Th2 cytokine expression. In mice, it directly induces macrophages to produce IL-12 and inhibits IL-10 expression by LPS-stimulated macrophages (Ashkar et al., 2000).

Table 1. Statistical analysis of avian macrophage microarray (AMM) data

Item	Amount
Elements on the AMM	4,906
Elements with high-quality replicate data	3,140
Elements exhibiting >2-fold change after *Eimeria acervulina* treatment	288
Statistically significant >2-fold changes after *E. acervulina* treatment	111
Elements exhibiting >2-fold change after *Eimeria maxima* treatment	262
Statistically significant >2-fold changes after *E. maxima* treatment	134
Elements exhibiting >2-fold change after *Eimeria tenella* treatment	282
Statistically significant >2-fold changes after *E. tenella* treatment	122
Total number of unique elements exhibiting significant expression changes	265

The paradigm of Th1-Th2 cytokine polarization suggests that early expression of Th1 cytokines is critical to a protective host response against intracellular infection (Abbas et al., 1996). Therefore, factors (including antigens) augmenting Th1 and inhibiting Th2 cytokine expression might function as powerful modulators of cell-mediated immunity, the main effector branch of the immune system of the bird against coccidiosis (Lillehoj et al., 2004). In our study using a quantitative RT-PCR, many cytokines involved in Th1 and Th2 pathways were induced simultaneously early after infection, reflecting the complexity of the local immune response induced by *Eimeria* in the intestine (Hong et al., 2006b,d). In contrast, other genes such as IL-16 and quiescence-specific protein were consistently repressed in macrophages after infection with *Eimeria*. Quiescence-specific protein is a secreted 20-kDa molecule belonging to the lipocalin protein family and among the most prevalent proteins present in quiescent chicken heart mesenchymal cells (Bédard et al., 1987) and chick embryo fibroblasts (Mao et al., 1993). By contrast, this protein is virtually absent in actively dividing cells. However, during intracellular infection of chick embryo fibroblasts by herpes virus, Morgan et al. (2001) observed high expression of the protein, suggesting that the virus inhibits cell cycle progression while allowing those cells to accumulate factors needed for its own replication. Interestingly, other cytokine and chemokine elements have been differentially expressed following treatment with the different *Eimeria* species, including K60 and IFN-γ; K60 has been shown to be highly induced at 48 h in *E. tenella*-exposed macrophages but repressed at 48 h in *E. acervulina*- and *E. maxima*-treated macrophages. In addition, IFN-γ has also been shown to be highly expressed in *E. tenella*-treated birds but not in chickens exposed to the other parasites. In vivo K60 transcripts levels have been shown to remain unchanged or increase slightly compared with levels of other chemokines (MIP-1β and K203) following *E. tenella* or *E. maxima* infections (Laurent et al., 2001). Increased IFN-γ levels in response to such infections are well documented both in vitro (Lillehoj and Choi, 1998; Yun et al., 2000) and in vivo (Laurent et al., 2001; Min et al., 2003; Hong et al., 2006b,d), especially in the early response to *Eimeria*.

Following treatments with different *Eimeria* species, IL-18 and MIP-1β have also shown differential gene expression; IL-18 has been induced at 18 h in *E. acervulina*- and *E. tenella*-treated macrophages but only after 48 h in response to *E. maxima* exposure. Expression of MIP-1β has been shown to peak at 18 h in response to *E. acervulina* and *E. tenella* sporozoites, but it has been shown to induce at its highest level at 4 h postinfection with *E. maxima*. Further, although little is known about the chemokine ah221, it is noteworthy that it has been upregulated very early on after infection with all 3 *Eimeria* infections, albeit at a much higher level in *E. acervulina*, and the transcript levels have been shown to decrease progressively with time. In summary, although a shared similarity in transcript quality exists among the 3 *Eimeria* parasites using the AMM, a difference remains obvious in the magnitude, direction, and timing of the immune responses to each individual species. Preliminary studies using the CIELA to assess local transcriptional changes following coccidio-

Table 2. *Eimeria*-induced immune-related gene expression changes

	Sporozoite treatment			
Gene	*Eimeria acervulina*	*Eimeria maxima*	*Eimeria tenella*	Measurement[5]
Interleukin-1β	++	++	++	Q RT-PCR
Interleukin-6	+	++	+	Q RT-PCR
Interleukin-18	++	+	+	Array, Q RT-PCR
Chemokine K60	—	—	++	Array, Q RT-PCR
Chemokine K203	++	++	++	Array, Q RT-PCR
Chemokine ah221	+	+	+	Array
Macrophage inflammatory protein-1β	+	+	++	Array
Interferon-γ	—	—	++	Q RT-PCR
Inducible NO synthase	+	++	++	Q RT-PCR

[1]Q RT-PCR = quantitative reverse transcription-PCR.

+ = 2.5-fold induction; ++ = >5-fold induction; — = repression.

Table 3. Twenty most highly induced annotated genes following primary and secondary *Eimeria maxima* infections

Access no.	Clone ID	Fold change	Gene name
Primary *E. maxima* infection			
CD729931	15Aug01_B24_1GAL88_084.ab1	34.48	Homologue to actin-like protein 6A
CD737984	16May01_F14_1GAL55_024.ab1	31.52	Weakly similar to Fed tick salivary protein 6
CD728749	10Jun01_N16_1GAL66_068.ab1	22.67	Melanocyte prolifeating gene 1
CD731103	26Jan01_G11_1GAL7_075.ab1	19.05	Otoancorin
CD734414	7Mar01_N20_1GAL51_035.ab1	17.86	Weakly similar to Kruppel-like factor 5 (intestinal)
CD729781	30Jun01_P21_1GAL87_060.ab1	17.62	ATP-binding cassette, subfamily B (MDR/TAP)
CD730632	7Jan01_K05_1GAL10_077.ab1	16.57	Farnesyltransferase CAAX box β
CF075018	6Jun01_K22_1GAL70_073.ab1	16.39	Eukaryotic translation initiation factor 3 subunit 4 (eIF-3 δ)
CD733572	8Dec00_K04_1GAL17_073.ab1	15.58	Serine protease HTRA1 precursor
CD733723	22Mar01_J03_1GAL50_049.ab1	14.56	NECAP endocytosis associated 2
CD734538	27Apr01_P04_1GAL52_075.ab1	14.52	Lymphoid-restricted membrane protein
CD731397	7May01_K18_1GAL38_101.ab1	12.35	Rho guanine nucleotide exchange factor 12 (leukemia-associated RhoGEF)
CD732440	20Jan01_A14_1GAL8_017.ab1	10.65	Multiple transmembrane domain protein
CD729631	18Jul01_F21_1GAL85_039.ab1	8.79	WD repeat domain 3
CD736504	21Jun01_P20_1GAL79_044.ab1	8.67	Regulator of nonsense transcripts 1
CD730957	27Mar01_I01_1GAL12_008.ab1	8.51	Kinesin-related microtuble-based motor protein
CD735000	27Jan01_I09_1GAL1_037.ab1	8.27	Ran binding protein 11
CD730496	7Jan01_H21_1GAL10_044.ab1	7.23	Putative pseudouridylate synthase specific to ribosomal small subunit precursor
CD731427	7May01_L10_1GAL38_074.ab1	6.98	Transcription factor (p38 interacting protein)
CD737628	21Feb01_A18_1GAL48_082.ab1	6.96	Pyridine nucleotide-disulfide oxidoreductase family protein
Secondary *E. maxima* infection			
CD731548	2Aug01_G05_1GAL71_078.ab1	32.41	Ig rearranged λ-chain mRNA V-region
BQ484926	pmp1c.pk007.e22	28.63	Erythrocyte band 7 integral membrane protein (stomatin)
CD727804	3Jun01_H07_1GAL63_016.ab1	17.86	Inner nuclear membrane protein Man1
CD735947	8Jun01_O04_1GAL72_075.ab1	17.06	Kelch-like 15
CD733710	22Mar01_I17_1GAL50_072.ab1	16.63	Amnesiac neuropeptide precursor
CD734167	24Dec00_H06_1GAL19_096.ab1	16.02	Similar to heat shock 70 kDa protein 4-like
CD738829	25July01_F14_1GAL59_023.ab1	12.75	PR-domain Zn finger protein 4
CD734924	27Jan01_N02_1GAL1_024.ab1	10.16	Inorganic pyrophosphatase (phosphohydrolase)
CD736434	21Jun01_K18_1GAL79_101.ab1	9.34	Pogo transposable element with ZNF domain isoform 1
CD731379	7May01_H09_1GAL38_048.ab1	9.24	Homologue to CLA4 (fragment)
CD729781	30Jun01_P21_1GAL87_060.ab1	8.73	ATP-binding cassette, subfamily B (MDR/TAP), member 4
CD737831	28July01_D20_1GAL54_029.ab1	8.25	WD repeat domain phosphoinositide-interacting protein 2
CD731103	26Jan01_G11_1GAL7_075.ab1	7.76	Otoancorin
CF075085	28July01_M05_1GAL54_082.ab1	6.74	CD81 antigen (target of antiproliferative antibody 1)
CD734811	27Jul01_C12_1GAL56_092.ab1	6.62	Presenilin 1
CD729201	26Jun01_P11_1GAL83_092.ab1	6.61	Homologue to peroxidase
CD730166	13Jul01_L17_1GAL89_089.ab1	6.15	Similar to osteoclast inhibitory lectin isoform 1
CD736607	21Jun01_D23_1GAL79_078.ab1	5.83	Homologue to autoantigen
CD738087	20Jul01_F18_1GAL57_087.ab1	5.68	Tubulin ε chain
CD727013	19May01_N23_1GAL60_084.ab1	5.50	Hydroxyprostaglandin dehydrogenase 15-(NAD)

sis have revealed that 1,120 elements have been significantly modulated, confirming the results from the AMM. These studies using functional genomics clearly indicate the power of applying new molecular tools to investigate complex interactions between host and pathogen.

APPLICATION OF FUNCTIONAL GENOMICS TO SALMONELLOSIS

Salmonellosis is complex disease involving many different components of host immunity (Monack et al., 2004). Because different serovar of *Salmonella* elicit different cellular and humoral immune responses, understanding the pathogenesis of this microorganism and developing new strategies against salmonellosis will require comprehensive investigation of the cellular and molecular events that occur during the invasion, survival, and induction of protective immunity. Functional genomics offers a powerful tool in this regard to assess changes in immune-related gene expression on a global basis. As of 2006, however, few studies have been reported describing alterations in gene expression during avian salmonellosis. Van Hemert et al. (2006) compared gene expression profiles in the intestines of *Salmonella*-infected chicken lines with differing growth rates and found that faster-growing broiler chickens showed enhanced expression of T cell activation-related genes, whereas slow-growing broiler chickens demonstrated macrophage activation-related genes at 1 d postinfection. Recently, we investigated transcriptional profiling of avian macrophages following in vitro infection with *Salmonella* with the goal of characterizing the cellular and molecular events that occur during *Salmonella* Enteritidis persistence in chickens. Using the AMM, the transcriptional profiles of HD11 cells infected with nalidixic acid-resistant *Salmonella* Enteritidis were analyzed at 2, 5, and 24 h postinfection. Out of 4,906 array elements, 338 genes exhibited 2-fold increases or decreases in expression ($P < 0.001$). Table 5 shows the 10 most representative annotated elements that were altered at the 3 time points. The chemokine ah294 consistently

Table 4. Twenty most highly repressed genes following primary and secondary *Eimeria maxima* infections

Access no.	Clone ID	Fold change[1]	Gene name
Primary *E. maxima* infection			
CD734924	27Jan01_N02_1GAL1_024.ab1	–100.00	Inorganic pyrophosphatase (pyrophosphate phosphorhydrolase)
CD730488	7Jan01_H16_1GAL10_060.ab1	–61.02	C21orf70 isoform B protein
CD733803	11Dec00_E07_1GAL18_006.ab1	–47.88	Zinc finger protein 147 (tripartite motif protein 25)
CD736598	21Jun01_D16_1GAL79_062.ab1	–46.07	D-Amino-acid oxidase
CD729849	30Jun01_C24_1GAL87_093.ab1	–28.16	Similar to Wdr22 protein
CD737726	28July01_G17_1GAL54_078.ab1	–23.38	Cell growth-regulating nucleolar protein
CD737698	21Feb01_G10_1GAL48_060.ab1	–20.38	Elongation factor Tu GTP binding domain containing 2
CD729243	26Jun01_B21_1GAL83_038.ab1	–18.38	Leucine-rich repeat Ser-Thr-protein kinase 1
CD734499	7Mar01_B22_1GAL51_053.ab1	–18.02	Weakly similar to divalent cation tolerant protein CUTA
CD733052	20Mar01_J21_1GAL32_049.ab1	–17.78	Polymerase (DNA directed), λ
CD737238	10Mar01_G18_1GAL46_094.ab1	–15.59	Heme carrier protein 1
CD737814	28July01_A14_1GAL54_020.ab1	–14.74	Topoisomerase (DNA) I
CF075105	25July01_C13_1GAL59_012.ab1	–14.52	Homologue to ribosomal protein S9
CD737375	19Feb01_C12_1GAL47_090.ab1	–13.23	Insulin induced gene 1
CD738012	16May01_M19_1GAL55_019.ab1	–12.94	Solute carrier family 13 (Na-sulphate symporters), member 1
CD737004	25Mar01_E15_1GAL44_038.ab1	–12.58	Complement component 1, q subcomponent, α polypeptide
CD739377	17Aug01_F18_1GAL95_086.ab1	–10.81	Oligonucleotide-oligosaccharide-binding fold containing 2A
CD738023	16May01_N05_1GAL55_084.ab1	–10.43	Glucose-6-phosphate 1-dehydrogenase
CD736219	26July01_C09_1GAL73_044.ab1	–10.38	Homologue to AT5g11000/T30N20_270
CD729115	26Jun01_H05_1GAL83_080.ab1	–9.97	Homologue to ribulose-phosphate 3-epimerase
Secondary *E. maxima* infection			
CD737917	28July01_D02_1GAL54_029.ab1	–100.00	Chronic lymphocytic leukemia deletion region gene 6 protein
CD732828	5Jan01_L17_1GAL9_090.ab1	–60.74	SPFH domain family, member 2
CD737329	19Feb01_G18_1GAL47_092.ab1	–43.18	Brix domain containing 2
CD732108	19Jan01_L24_1GAL6_094.ab1	–34.44	Heterogeneous nuclear ribonucleoprotein R
CD732607	20Apr01_P18_1GAL31_103.ab1	–29.52	NADH dehydrogenase subunit II
CD734122	24Dec00_M10_1GAL19_067.ab1	–28.78	Transmembrane protein 45b
CD737331	19Feb01_H01_1GAL47_012.ab1	–22.82	Glycolate oxidase Fe-S subunit putative
CD733508	8Dec00_G14_1GAL17_030.ab1	–21.21	Proteasome (prosome, macropain) 26S subunit, non-ATPase, 3
CD735479	6Jun01_A18_1GAL70_085.ab1	–21.12	Solute carrier family 35, member C2
CD735313	8Jan01_L09_1GAL3_046.ab1	–19.51	Complement receptor 1
CD733028	20Mar01_J04_1GAL32_065.ab1	–18.14	Pyruvate dehydrogenase (lipoamide) β
CD738344	30July01_P23_1GAL58_091.ab1	–16.37	Protein Tyr phosphatase-like A domain containing 1
CD733883	11Dec00_O22_1GAL18_075.ab1	–14.61	U1-snRNP binding protein homolog
CD731155	6Mar01_B12_1GAL13_085.ab1	–13.73	I κ-B kinase ε
CD737209	19Mar01_P10_1GAL45_075.ab1	–13.10	Bromodomain containing 7
CD731768	29Jun01_E05_1GAL86_071.ab1	–12.92	Epoxide hydrolase 2, cytoplasmic
DN829822	pat.pk0028.h3.f	–12.49	MHC class II-associated invariant chain
CD736598	21Jun01_D16_1GAL79_062.ab1	–11.90	D-amino-acid oxidase
CF075077	28July01_G23_1GAL54_078.ab1	–11.60	Ribosomal protein S6
CD737714	28July01_J12_1GAL54_097.ab1	–11.27	RAD21 homolog

[1]Negative value means a downregulation.

showed the highest expression at all time points examined in the *Salmonella* Enteritidis-infected HD11 cells. Additionally, ah294 is a CC chemokine that activates innate immune responses and prevents the apoptosis of virus-infected mammalian macrophages (Tyner et al., 2005). Also, CLN8 may play a role in protein secretion during immune activation (Winter and Ponting, 2002), and its defect has been associated with neuronal ceroid lipofuscinosis 8 disease, which is characterized by the accumulation of ATP synthase subunit C. Other immune-related genes with enhanced gene expression include immune-responsive protein 1, IL-6, inducible T cell costimulator, antiapoptotic NR13, matrix metalloproteinase 9, and glutamate-Cys ligase. The antiapoptotic NR13 protects cells from apoptosis by counteracting the proapoptotic protein BAX in chickens (Lee et al., 1999). Glutamate-Cys ligase is a rate-limiting enzyme for glutathione synthesis and is associated with oxidative stress reduction as well as NO production (Murphy et al., 1991).

Table 5 also shows that many genes associated with transcription, cell adhesion, and cell proliferation were downregulated in HD11 macrophages following *Salmonella* Enteritidis infection. These include β-catenin, placental growth factor (**PGF**), ring finger protein 19 (**RFP19**), thymosin-β4, SP1, and inhibitor of κB kinase ε. β-Catenin is a subunit of the multiprotein cell-cell adhesion complex containing E-cadherin, α-catenin, and γ-catenin that activates target genes of the Wnt signaling pathway and regulates cell adhesion and permeability at intercellular junctions (Ratcliffe et al., 1997). Expression of PGF and thymosin-β4 is involved in cell adhesion, proliferation, and differentiation, and the deficiency of PGF has been associated with a diminished and abbreviated inflammatory response in the mouse (Oura et al., 2003). Additionally, SP1 and RFP19 are major transcription factors involved in the TGF-β signaling pathway that lead to angiogenesis, immunosuppression, and apoptosis, and their expression may lead to the inhibition of local immunopathology (Li et al., 2006). Gallinacin 1 (β-defensin 1) has also been downregulated in HD11 following infection with *Salmonella* Enteritidis, although this gene has been

Table 5. Ten most highly induced and repressed genes following *Salmonella* infection of chicken HD11 macrophages

	Upregulated genes			Downregulated genes		
HPI[1]	GenBank accession no.	Fold change	Gene name	GenBank accession no.	Fold change[2]	Gene name
2	CK612928	272.9	Chemokine ah294	CK615438	–12.1	β-Catenin
	CK613680	217.4	Chemokine (C-C motif) ligand 20	BM487268	–10.9	Placental growth factor
	CK613753	101.9	Chemokine K60	CK608255	–8.9	Hypothetical protein xp_429578
	CK614751	72.27	Hypothetical protein dkfzp434m1616.1	BM487890	–8.8	Frizzled homolog 9 (drosophila)
	CK615207	62.11	Hypothetical protein, clone 1h23	CK607525	–8.7	Ras homolog gene family, member A
	CK613425	42.59	Ceroid-lipofuscinosis, neuronal 8	CK607523	–7.2	Abhydrolase domain containing 12 (ABHD12)
	CK613517	37.63	Immune-responsive protein 1	CK611364	–6.4	Chloride intracellular channel 2 (CLIC2)
	CK613692	25.93	Interleukin-6	CK606789	–5.9	Transcription factor SP1
	CK615246	25.24	L-Amino acid oxidase	CK614627	–5.7	Ring finger protein 19
	CK613244	21.58	Inducible T cell costimulator	CK614834	–5.5	Inhibitor of κ-B kinase epsilon
5	CK612928	574.9	Chemokine ah294	CK615438	–13.7	β-Catenin
	CK613680	193.2	Chemokine (C-C motif) ligand 20	CK608255	–12.6	Hypothetical protein xp_429578
	CK615115	101	Glutamate-Cys ligase, modifier subunit	BM487268	–11.2	Placental growth factor
	CK615246	89.07	L-Amino acid oxidase	BQ484520	–10.4	Gallinacin 1
	CK614751	83.6	Hypothetical protein dkfzp434m1616.1	CK607525	–10.4	Ras homolog gene family, member A
	CK615207	76.85	Hypothetical protein, clone 1h23	BM487890	–9.5	Frizzled homolog 9 (drosophila)
	CK613753	76.72	Chemokine K60	CK607523	–8.5	Abhydrolase domain containing 12 (ABHD12)
	CK613425	50.94	Ceroid-lipofuscinosis, neuronal 8	CK614627	–7.4	Ring finger protein 19
	CK609652	23.32	Antiapoptotic NR13	CK611364	–6.9	Chloride intracellular channel 2 (CLIC2)
	CK613692	21.26	Interleukin-6	CK606789	–6.8	Transcription factor SP1
24	CK612928	991.2	Chemokine ah294	BQ484520	–16.3	Gallinacin 1
	CK615115	101.8	Glutamate-Cys ligase, modifier subunit	CK608255	–13.1	Hypothetical protein xp_429578
	CK614751	74.88	Hypothetical protein dkfzp434m1616.1	CK615438	–9.3	β-Catenin
	CK615207	50.67	Hypothetical protein, clone 1h23	CK614834	–8.1	Inhibitor of κ-B kinase ε
	CK613425	47.89	Ceroid-lipofuscinosis, neuronal 8	CK607331	–7.6	Thymosin-β4
	CK613753	44.92	Chemokine K60	BQ037941	–6.4	Myeloproliferative leukemia virus oncogene
	CK607070	35.04	Matrix metallopeptidase 9	CK614627	–5.3	Ring finger protein 19
	CK613244	26.85	Inducible T cell costimulator	BQ484683	–5.2	Proteasome (prosome, macropain) subunit, α type, 5
	BQ484885	20.01	Stearoyl-CoA desaturase (δ-9-desaturase)	BQ484967	–5.0	Pericentriolar material-1 (PCM-1 gene)
	CK609652	17.16	Antiapoptotic NR13	CK607342	–5.0	Heparanase

[1]HPI = hours postinfection.

[2]Negative value means a downregulation.

reported to be induced by *Salmonella* Enteritidis infection in cultured chicken vaginal cells (Yoshimura et al., 2006).

In conclusion, global transcriptional profiling analysis of *Salmonella* infection in chicken macrophages showed that many different genes are involved in the host response to *Salmonella*, and further studies are needed to better understand pathogen survival strategy and *Salmonella* persistence inside the host. Furthermore, integrated analysis of both in vivo local gene expression using the CIELA and in vitro transcription changes of immune-related genes in chicken macrophages following *Salmonella* infection needs to be carried out to obtain a better understanding of host-pathogen immunobiology in salmonellosis.

CONCLUSIONS

In the poultry industry, there are mounting concerns over increasing governmental regulations of the use of in-feed antibiotic growth promoters, which historically have been used to raise poultry under high density housing conditions. In addition, questions concerning the ability of current vaccines to adequately protect against emerging hypervirulent strains of pathogens and a lack of suitable, cost-effective adjuvants are raising new interest in the development of alternative control strategies against poultry pathogens such as *Eimeria* and *Salmonella*. Comprehensive investigation of the immunobiology of host-pathogen interactions in coccidiosis and salmonellosis must precede the development of novel effective alternative control strategies for these pathogens. Application of immunogenomic and proteomic tools to enteric disease research is critical not only to enhance our understanding of basic immunological mechanisms of the avian host but also to develop efficient vaccination protocols against pathogens of economic importance to the poultry industry.

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